Journal of Surgical Oncology 1997;65:159–163

p53 Tumor Suppressor Protein and Tissue Proliferative Fraction in Infiltrating Duct Carcinoma

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Background and Objectives: Breast cancer continues to frustrate oncologists worldwide. In India, it is the second most common neoplasm among women and is increasing in incidence. Several molecular markers have been associated with a poor prognosis in patients with breast cancer, and the presence of these markers is often thought to provide information on the biological behavior of the malignant breast tumor. Much attention has recently been focused on the tumor suppressor gene p53. Mutation or alteration in this gene leads to the loss of negative growth regulation and hence to rapid cell proliferation. The present study was designed to evaluate the connection between expression of the p53 protein and its relation to the tissue proliferative compartment as measured by expression of the proliferating cell nuclear antigen (PCNA).

Methods: Expression of p53 and PCNA were detected by immunocytochemistry in paraffin-embedded sections of infiltrating duct carcinoma and control breast tissue (normal tissue and adenoma).

Results: A significant correlation was observed between expression of p53 and PCNA. A significant correlation was also observed between expression of p53 and grade of tumor and stage of disease.

Conclusions: Our results support the hypothesis that accumulation of p53 is associated with a high tumor proliferation rate an association that might be expected in view of the role of wild p53 as a negative regulator of cell proliferation. *J. Surg. Oncol. 1997;65:159–163.*

KEY WORDS: **p53; PCNA; proliferation; breast cancer**

INTRODUCTION

Breast cancer is the second most common female cancer in India as is evident from cancer registry data in Trivandrum, Bangalore, Delhi, and Madras. It is the leading cancer in Bombay [1], where a rise in the age-adjusted incidence rate of breast cancer in women from 17.9 to 24.9 per 100,000 population between 1965 and 1985 is reported [2]. In addition, a rise has been projected in the total number of female breast cancer cases in India from 60,000 in 1991 to 80,000 by the year 2001 [3]. Most decisions concerning the use of adjuvant therapy are currently based on the size of the tumor and involvement of lymph nodes. This, however, is often not an accurate predictor of relapse and survival [4] and is highlighted by a report that ~30% of breast cancer patients who are node

Contract grant sponsor: Kerala State Committee on Science, Technology, and Environment; Contract grant number: G.O. (MS) No. 15/96/STED.

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Accepted 10 April 1997

negative are considered to have recurrence within the first 5 years of follow-up [4]. The ability to predict which patients are susceptible to relapse would, therefore, enable oncologists suitably to alter postoperative management, leading ultimately to higher survival rates.

Evidence generated in recent years has begun to unveil the genetic mechanism of oncogenesis. Much attention has recently been focused on the role of the tumor suppressor gene p53 in breast cancer. Mutations of the p53 gene constitute the most common change seen in breast cancer patients, and frequency rates range from 15% to 50% [5–10]. The 20 kb p53 gene located on chromosome 17 (p13) encodes for a 53 kd nuclear phosphoprotein. It functions as a negative regulator of cell growth and also inhibits transformation [11]. Mutation or alterations in the gene lead to the loss of this negative growth regulation and hence more rapid cell proliferation [12]. Moreover, since p53 functions to control entry or progression through the cell cycle, it is expected that tumors with an inactivated p53 would have a higher rate of proliferation. In the present study, immunocytochemistry was used to evaluate the relationship between expression of p53 protein and its relation to the tumor cell proliferation rate as measured by proliferating cell nuclear antigen (PCNA) expression. PCNA is a 36 kd acidic nonhistone protein and has been identified as an auxiliary protein of DNA polymerase delta. It has been demonstrated to play a critical role in the initiation of DNA replication and cell proliferation. As a result, its level of synthesis and expression correlates directly to the rates of proliferation and DNA synthesis, particularly in the late G1 and S phase [13].

MATERIALS AND METHODS

Study Subjects

A total of 118 subjects were analyzed in the study, including 68 samples of infiltrating duct carcinoma and 50 control tissue. Breast tissue samples were obtained from patients undergoing lumpectomy and mastectomy. Control breast tissue was dissected out from mastectomy specimens and from fibroadenomas. Distribution of analyzed subjects is given in Table I.

Immunocytochemistry for p53 and PCNA Expression

Sections stained with hematoxylin and eosin were evaluated for each patient, and a representative paraffin block was selected for immunohistochemical studies. Deparaffinized sections were rehydrated through graded ethanol to distilled water. All sections were processed for antigen retrieval [14]. For this, sections were placed in prewarmed Target Unmasking Fluid (TUF, Kreatech, Amsterdam, The Netherlands) at 90°C for 10 minutes and allowed to cool to room temperature, followed by quenching of endogenous peroxidase with 0.3% H_2O_2 and blocking of nonspecific binding with 3% bovine serum albumin (BSA). Sections were incubated overnight with the respective primary monoclonal antibody, (p53 DO 7 and PCNA - PC10, (Dako AS, Glostrup, Denmark) at 4°C. Negative control was carried out by omitting the primary antibody. The reaction was visualized using a streptavidin-biotin-immunoperoxidase system (Dako AS), with diaminobenzedine as chromogen. All sections were then counterstained with haematoxylin.

Evaluation of p53 and PCNA Immunostaining

Immunocytochemical staining for p53 was considered significant when characteristic nuclear staining was seen in >20% of the cells. PCNA expression was also regarded as significant when observed in >20% of the cells. In addition, an expression index was created. This was done by classifying the protein expression into three categories based on the number of cells with positive expression. Thus grade 1 expression included those samples with <20% expression, grade 2 included samples showing 21–50% expression, grade 3 included samples exhibiting positive expression in >50% of cells. The concomitant expression of p53 and PCNA in different tissue samples was statistically analyzed by Spearman correlation tests.

RESULTS

p53 and PCNA positivity was apparent from clear nuclear staining in tumor cells (Figs. 1A, B; 2A, B). Expression of p53 was limited to tumor tissue and was absent in normal and control samples, including the seven cases of fibroadenoma (Tables II and III). A clear increase in PCNA and p53 staining was seen in cases compared with adjacent noncancerous epithelial cells. Cytoplasmic staining for p53 was not found in any of the samples analyzed. p53 was mainly found to be localized in the nuclear and perinuclear areas. A significant statistical correlation was observed between p53 expression and grade of the tumor. Of the 68 cases of infiltrating ductal carcinoma, 42 had grade 2 tumors and 26 had grade 3. p53 was found to be expressed in 38 cases with grade 2 tumors (90%). Similar results were obtained for PCNA in grade 2 tumors. Grade 3 tumors also had a significant expression of p53 and PCNA. Expression of p53 and PCNA was found to increase as tumors progress to a state of poor differentiation. Labelling index of PCNA had a positive correlation to expression of p53 ($r = 0.62280$, $P = 0.00002$). In most of the cases analyzed, accumulation of p53 protein resulted in an increase in the PCNA staining. p53 and PCNA expression was also found to have a correlation with TNM staging ($r = 0.057207$, $P = 0.00012$). Expression of p53 and PCNA was found to be more in stage 3 than in stage 2 tumors. The percentage of positivity of p53 and PCNA were also found to increase with increase in tumor size.

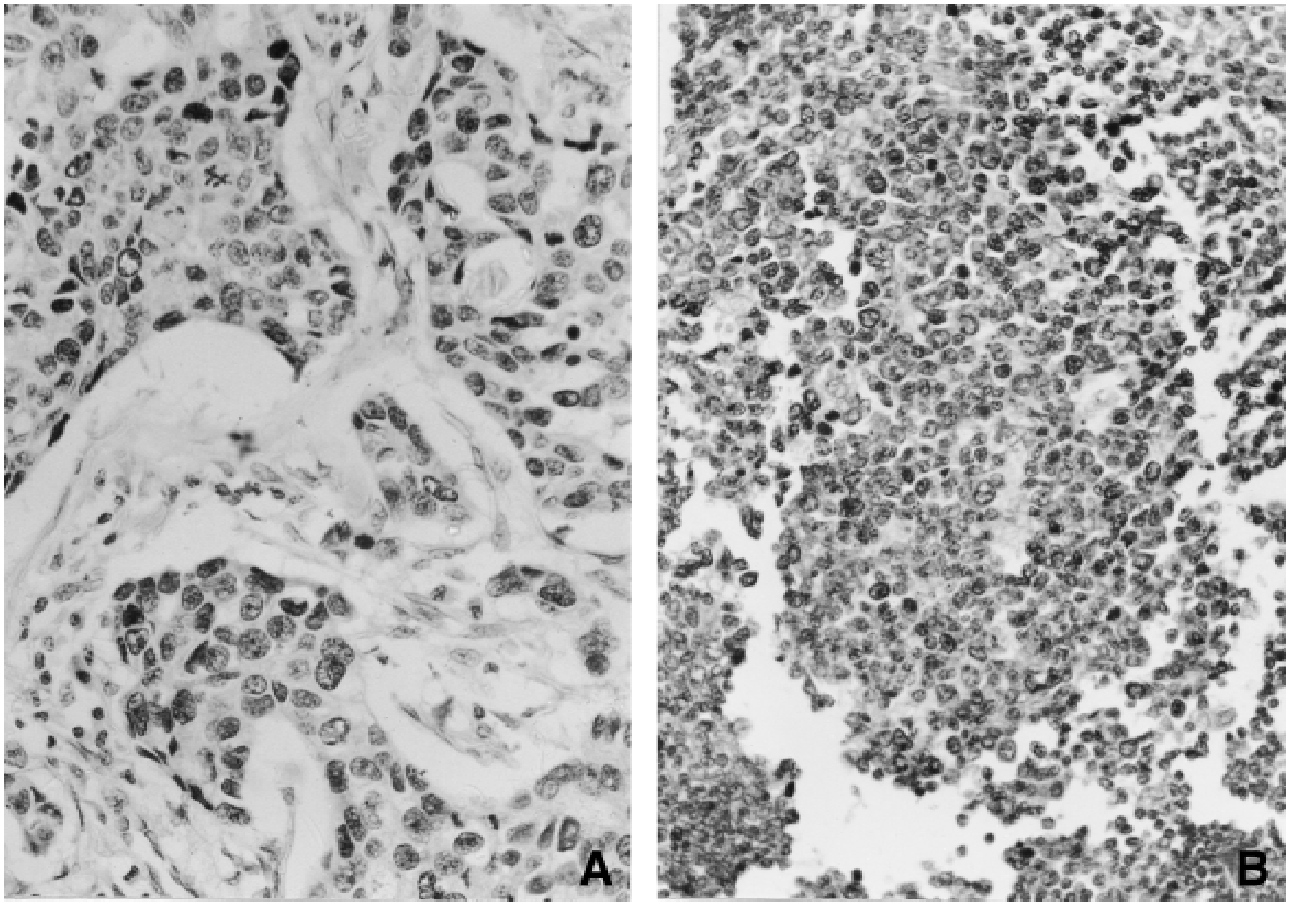

Fig. 1. **(A)** Typical nuclear staining pattern for PCNA seen in infiltrating duct carcinoma. **(B)** Typical nuclear staining for PCNA in the corresponding axillary lymph node.

TABLE I. Subjects Analyzed in the Study

Tissue	Number
Normal breast tissue	30
Fibroadenoma	20
Infiltrating ductal carcinoma Grade 2	42
Infiltrating ductal carcinoma Grade 3	26

DISCUSSION

Breast cancer is an unpredictable disease. The natural history and prognosis of breast cancer patients varies considerably from patient to patient, and even very small malignant lesions at the limit of detection by mammography or palpation may have metastatic potential. At present, most decisions concerning the use of adjuvant therapy is based on the size of the tumor and involvement of lymph nodes. This, however, is often not an accurate predictor of relapse and survival [4]. Therefore, the ability to predict which patients are susceptible to relapse would enable the physician suitably to alter the course of postoperative management, ultimately leading to higher survival rates. The aggressiveness of a solid tumor is very often influenced by a number of molecular factors in the primary tumor. Staging or grading the tumor at the molecular level may thus provide information on proliferation, the treatment response, and invasiveness of tumor. This is emphasized by the recent call by The American Joint Committee on Cancer for the inclusion of biological markers in addition to those of anatomic and pathological extent for classifying cancer [15]. The present study was designed to determine such a role for p53 and PCNA. The study was based on the premise that lesions with immunohistochemically detectable p53 would have higher rates of proliferation. Mutant p53 protein accumulates in high concentrations, due to its prolonged half life and accumulation of protein in the nucleus. Thus detection of an abnormal accumulation of p53 has been thought to be an indirect evidence of a mutation [16,17].

Previous studies that have examined the relationship between p53 mutation and cell proliferation in breast cancer show conflicting data. Our results agree with that of Allred et al. [16], who reported accumulation of p53 measured by immunocytochemistry to be associated with a high tumor proliferation rate. A direct relationship be-

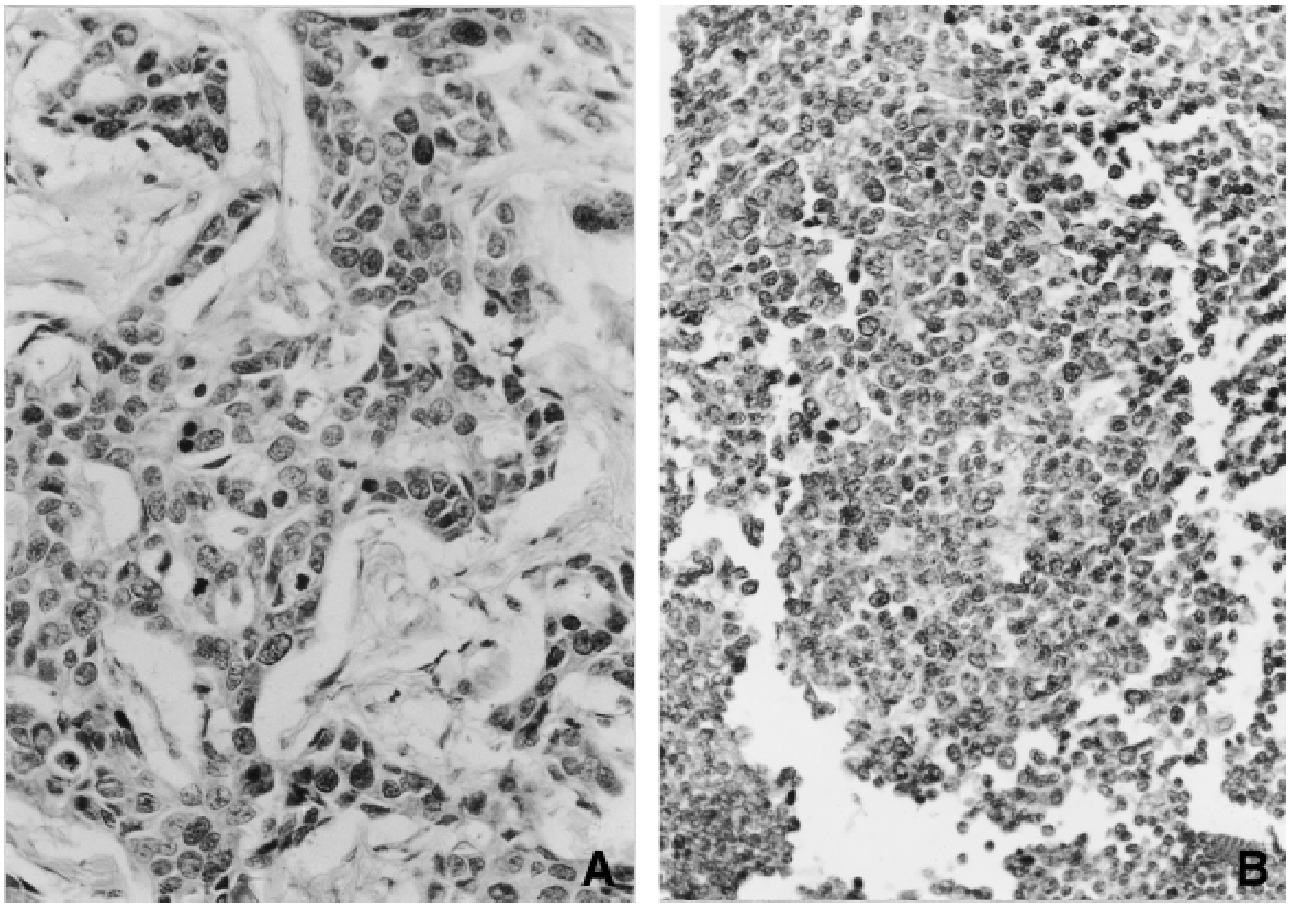

Fig. 2. **(A)** Typical staining of p53 protein in infiltrating ductal carcinoma. **(B)** Typical nuclear staining for p53 in the corresponding axillary lymph node.

TABLE II. Expression of p53 in Study Population

Tissue	No. samples showing positivity for p53
Normal (n = 30)	0/30
Fibroadenoma (n = 20)	0/20
Infiltrating ductal carcinoma Grade 2 (n = 42)	38/42
Infiltrating ductal carcinoma Grade 3 (n = 26)	24/26

TABLE III. Expression of PCNA in the Study Population

Tissue	Expression of PCNA
Normal (n = 30)	24/30
Fibroadenoma (n = 20)	20/20
Infiltrating ductal carcinoma grade 2 (n = 42)	38/42
Infiltrating ductal carcinoma grade 3 (n = 26)	26/26

tween p53 and expression of Ki 67, a proliferation-associated antigen also has been reported [18], an association that might be expected in view of the role of normal p53 as a suppressor of cell proliferation. The present study also supports the hypothesis that p53 negatively regulates cell division and that mutations abrogate this regulation, giving rise to cells with a greater proliferative potential. Since p53 mutations are common in breast cancer, it has been thought that p53 mutations could be associated with more aggressive tumors or those with higher likelihood of occult distant metastasis [19]. Mutations in p53 are associated with its ability to transform cells in culture and loss of its usual negative control on cell proliferation. For these reasons, it is also believed that a mutation would predict a greater likelihood of early recurrence of breast cancer after primary surgical therapy [19]. Strong nuclear p53 accumulation is also often significantly correlated to several pathobiological variables, indicating an aggressive, genetically unstable, and rapidly proliferating tumor. We also observed that poorly differentiated tumors showed strong overexpression of p53. Similar results have been described earlier that show a higher grade to be correlated with p53 accumulation [20,21]. Breast cancer patients with tumors exhibiting a high S-phase fraction have been shown to respond to

adjuvant cytotoxic treatment [22]. It would be of interest to investigate whether tumors with p53 accumulation, which often exhibit high S-phase fraction, will also respond to such treatment. Thus p53 alterations may have a prognostic application especially for predicting future recurrence. These patients are now being followed up to see whether this is clinically valid.

ACKNOWLEDGMENTS

A.L.K. is supported by a Senior Research Fellowship from the Council of Scientific and Industrial Research, India.

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